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TECHNICAL MANUSCRIPT 620

PRELIMINARY STUDIES ON THE GROWTH OF CHIKUNGUNYA VIRUS USING BABY HAMSTER KIDNEY CELL SUSPENSION CULTURES

Jack L. Davis
Howard M. Hodge
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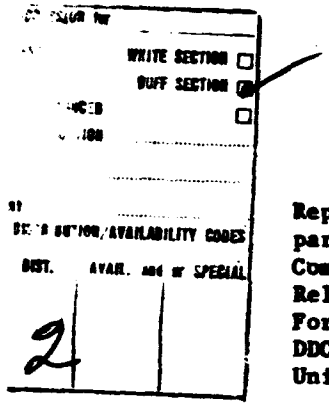

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Product Development Division
AGENT DEVELOPMENT & ENGINEERING LABORATORIES

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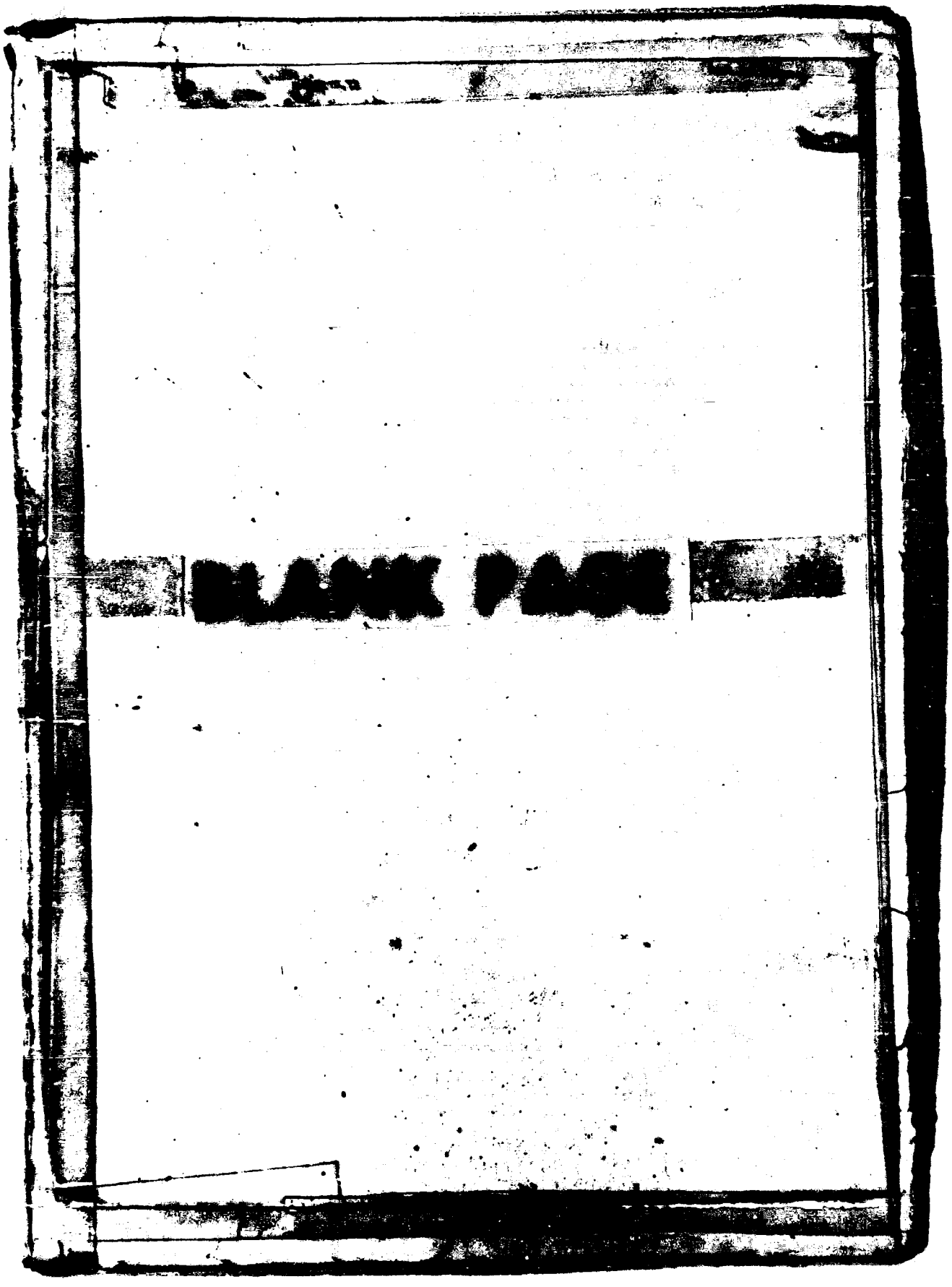
August 1970

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

ABSTRACT

This report describes the methods used to obtain high titers of chikungunya virus using suspension cultures of BHK-21 (clone 13) cells. The cells were grown at 37 C to a cell concentration of 1×10^6 to 2×10^6/ml. Following maximum cell growth, the cells were inoculated with chikungunya virus at a multiplicity of 1 to 2 $SMICLD_{50}$ per cell in the spent Eagle's minimum essential medium for suspension cultures (MEMS), or the cell cultures were centrifuged at 1,000 rpm and resuspended in either fresh MEMS or medium 199 prior to inoculation. The medium used had no effect on virus titer. The inoculated cultures were incubated at 34 C until the cell viability dropped to 30%, which usually occurred 28 to 30 hours post-inoculation. Following these procedures, chikungunya virus titers of 10.3 to 11.8 $\log_{10}$ $SMICLD_{50}$/ml were obtained.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I. INTRODUCTION*

Chikungunya virus was first isolated by Ross[1] during an outbreak of dengue-like fever in Tanganyika, East Africa, in 1953. Subsequent epidemics of dengue-like fever have been recorded in Thailand,[2,3] India,[4] Japan,[5] and Singapore.[6] Although several clinical and epidemiological studies have been made of the virus disease,[3,7-10] no information on the growth of this virus in submerged tissue culture is available. A survey of the literature revealed that monolayer cultures of chick fibroblast,[11] Vero,[12] and FL cells[13] have been used to establish growth curves of the virus. However, optimum parameters, such as cell concentration, multiplicity of infection, and incubation temperatures, have not been established to obtain maximum virus titers.

A preliminary study was made to select a cell line that would grow in suspension and produce high virus titers. It was found from these studies that BHK-21 (clone 13) cells (baby hamster kidney fibroblasts) adapted to grow in suspension cultures using the procedure described by Chapstick et al.[14] supported rapid growth and high titers of chikungunya virus. This report defines the procedures used to grow chikungunya virus in suspension cultures of BHK-21 (clone 13) cells.

II. MATERIALS AND METHODS

A. TISSUE CULTURE CELLS

The cell line used throughout this study was baby hamster kidney [BHK-21 (clone 13)] initially obtained from the American Type Culture Collection. The origin of BHK-21 has previously been described by Macpherson and Stoker.[15]

B. VIRUS SEED STOCK

A seed stock of chikungunya virus (Banganike strain)[16] was prepared in the form of a 10% suckling mouse brain suspension in medium 199 containing 5% calf serum. The seed was stored at -70 C until used.

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C. MEDIA

1. Eagle's Minimum Essential Medium

Eagle's minimum essential medium (MEM) was used to grow BHK-21 monolayers in 8- or 16-oz prescription bottles. This medium, as described by Merchant, Kahn, and Murphy,[17] consisted of 90% MEM made up with Hanks balanced salt solution fortified with 2X concentration of L-glutamine and 10% calf serum. Antibiotics were incorporated to give final concentrations of 100 units penicillin/ml, 100 μg of streptomycin/ml, and 20 units of Mycostatin/ml.

2. Eagle's Minimum Essential Medium for Suspension Cells

Eagle's minimum essential medium for suspension cells (MEMS) was prepared by omitting calcium salts from the MEM formula and increasing NaH_2PO_4 10-fold. Prior to use, 10% by volume of autoclaved tryptose phosphate broth (Difco) and 10% by volume of filtered calf serum were added. All suspension cultures were grown in the presence of 0.12% methylcellulose of 15 cp viscosity. The concentration of antibiotics used was the same as described for MEM.

3. Medium 199

Medium 199 was prepared as described by Merchant et al.[17] fortified with 5% calf serum and 0.12% methylcellulose (15 cp).

Cells were cultured in suspension in cylindrical Pyrex-jacketed Bellco spinners. The volume of culture used (400 ml) occupied about one-half of the vessel. The cultures were stirred by Teflon-coated bar magnets agitated by mechanically propelled external magnets. The cultures were incubated at 37 C.

D. VESSELS USED FOR VIRUS PRODUCTION

The vessels used for virus growth studies were the Pyrex-jacketed Bellco spinners or 1-liter Erlenmeyer shaker flasks containing 400 ml of medium. All virus-infected cells were maintained at 34 C.

E. ADAPTATION OF BHK-21 (CLONE 13) CELLS TO SUSPENSION CULTURE

BHK-21 (clone 13) cells that were grown in 8-oz prescription bottles in MEM at 37 C and had undergone an unknown number of monolayer transfers were used as starter cells for suspension cultures. The monolayer cultures were trypsinized and resuspended in 400 ml of suspension medium at a concentration of 5×10^5 cells/ml, then transferred to the jacketed Bellco spinner

vessels. The culture vessels were placed on a Bellco magnetic stirrer at 37 C. During the early stages of adaptation, cells clumped and attached to the glass. This was counteracted by trypsinizing the cells with 20 ml of a 5% stock solution of trypsin for each 100 ml of suspension culture. After trypsinization the cells were removed from suspension by centrifuging for 10 minutes at 1,000 rpm and resuspended in fresh medium at a concentration of 5×10^5 cells/ml.

In the early stages of adaptation the generation time was approximately 36 hours; however, after 30 days or 10 complete medium changes the generation time was about 24 hours. Following passages of the cells for 30 days in suspension cultures, a cell concentration of 1.5×10^6 to 2.2×10^6/ml was obtained after a 3-day growth period.

F. CELL PROPAGATION AND VIRUS PRODUCTION

Cells used for virus production were grown from an initial concentration of $5 \times 10^{5.0}$ cells/ml to a maximum concentration of 1.5×10^6 to 2.2×10^6 cells/ml in 3 days. The spinner cells were usually trypsinized and centrifuged at 1,000 rpm for 10 minutes. When fresh medium was used, the centrifuged cells were resuspended to a cell concentration of 1×10^6 to 2×10^6 cells/ml in 400 ml of either fresh MEMS or medium 199. The resuspended cells were transferred to either 400-ml Bellco spinners or 1,000-ml Erlenmeyer shaker flasks. To each 400 ml of resuspended cells, 0.8 ml of virus seed was added, giving an initial or zero-hour titer of approximately 2×10^6 to 3×10^6 $SMICLD_{50}$/ml. The pH of the growth medium was maintained within 7.2 to 7.4 by periodic additions of sterile 5% $NaHCO_3$ solution.

G. VIRUS ASSAY

Samples of chikungunya virus were diluted serially 10-fold in heart infusion broth (Difco). For LD_{50} titration, 0.03 ml of each dilution was inoculated intracerebrally into 1-day-old suckling mice; eight mice were used for each dilution. The LD_{50} value after a 10-day observation period was calculated by Reed and Muench's method.

III. RESULTS

Data for preliminary studies on the growth of chikungunya virus in BHK-21 suspension cells in different media are shown in Table 1. The data show no difference in the titer of virus growth in spent MEMS, fresh MEMS, or medium 199. Samples obtained at designated time intervals showed that maximal titers were obtained approximately 28 to 30 hours post-inoculation. Table 2 shows virus yield as a function of number of cells. The data indicate that a cell concentration of 1×10^6 to 2×10^6/ml should be used to obtain high yields of virus. Although the titers obtained using 4×10^6/ml are considerably higher, it was difficult to maintain the pH of 7.2 to 7.4 throughout the growth cycle. Table 3 shows virus titers of seven trials using a standard cell concentration of 2×10^6/ml. Using either MEMS or medium 199, the trials were harvested when the viability of the cells had dropped to 30%. Assays obtained for these seven trials indicate that chikungunya virus titers of 10.3 to 11.8 $\log_{10}$ $SMICLD_{50}$/ml can be obtained in 29 to 30 hours in BHK-21 cells.

TABLE 1. PRELIMINARY STUDIES OF YIELD OF CHIKUNGUNYA VIRUS IN BHK-21 SUSPENSION CELLS IN DIFFERENT MEDIA

Exp.	Medium	Number of BHK Cells per Milliliter	Hours After Inoculation	Virus Titer, $\log_{10}$ $SMICLD_{50}$/ml
1	Spent MEMS plus 10% calf serum	1.8×10^6	16	8.90
			24	10.10
			30	>10.00
			40	8.85
			48	9.01
	Fresh MEMS plus 10% calf serum	1.8×10^6	16	10.00
			24	10.30
			30	>10.00
			40	8.51
			48	9.05
2	199 plus 5% calf serum	1.14×10^6	21	9.80
			24	9.50
			28	10.90
	Fresh MEMS plus 10% calf serum	1.4×10^6	21	9.70
			24	10.10
			28	11.20

TABLE 2. EFFECT OF NUMBER OF CELLS ON VIRUS PRODUCTION

Exp.	BHK Cell Concn/ml in Medium 199	Age of Harvest, hours	Virus Titer, Log_{10} $SMICLD_{50}$/ml
1	2.5×10^5	24	8.70
	5.0×10^5	24	9.60
	1×10^6	24	>10.00
	2×10^6	24	>10.00
2	1×10^6	28	10.86
	2×10^6	28	11.12
	3×10^6	28	11.14
	4×10^6	28	11.51
	1×10^6	32	10.70
	2×10^6	32	10.50
	3×10^6	32	10.60
	4×10^6	32	11.00

TABLE 3. VIRUS HARVESTS USING STANDARDIZED BHK-21 CELL CONCENTRATION

Exp.	Medium Used	No. of BHK Cells/ml	Hours After Inoculation	Virus Titer, Log_{10} $SMICLD_{50}$/ml
1	Spent MEM plus 10% calf serum	2.1×10^6	30	11.8
2	199 plus 5% calf serum	2.1×10^6	28.5	11.8
3	199 plus 5% calf serum	2.1×10^6	28.5	11.6
4	199 plus 5% calf serum	2.0×10^6	28	11.1
5	199 plus 5% calf serum	2.0×10^6	30	10.5
6	Fresh MEMS plus 10% calf serum	2.0×10^6	34	10.4
7	Fresh MEMS plus 10% calf serum	2.0×10^6	34	10.3

IV. DISCUSSION

The foregoing experiments establish the feasibility of growing chikungunya virus in BHK-21 (clone 13) cell suspension. The virus-rich supernatant fluid, after centrifugation at 1,000 rpm to remove cells, offers a good starting material for purification and concentration studies because of the low content of non-viral material and high virus titer.

The use of BHK-21 (clone 13) cells in suspension cultures provides a simple and efficient method for virus propagation. Handling of cultures is minimal, because the medium has sufficient nutrients for the growth cycle of both the cells and the virus.

We found in this investigation that BHK-21 (clone 13) cells in suspension cultures produce a greater number of infectious virus particles per cell than monolayer cultures of chick fibroblasts[18] or FL cells.[13] A greater number of infectious particles is produced by the BHK cells, and virus titer is directly proportional to cell number, so a significant increase in virus yield per milliliter is obtained using high concentrations of BHK cells.

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13. ABSTRACT
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